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Axonal Regeneration into Acellular Nerve Grafts Is Enhanced by Degradation of Chondroitin Sulfate Proteoglycan

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Although the peripheral nerve has the potential to regenerate after injury, degenerative processes may be essential to promote axonal growth into the denervated nerve. One hypothesis is that the nerve contains growth inhibitors that must be neutralized after injury for optimal regeneration. In the present study, we tested whether degradation of chondroitin sulfate proteoglycan, a known inhibitor of axon growth, enhances the growth-promoting properties of grafts prepared from normal donor nerves. Excised segments of rat sciatic nerve were made acellular by freeze-killing before treatment with chondroitinase ABC. Chondroitinase-dependent neoepitope immunolabeling showed that chondroitin sulfate proteoglycan was thoroughly degraded throughout the treated nerve segments. In addition, neuronal cryoculture assays revealed that the neurite-promoting activity of acellular nerves was significantly increased by chondroitinase treatment. Control and chondroitinase-treated acellular nerves were then used as interpositional grafts in a rat nerve injury model. Axonal regeneration into the grafts was assessed 4 and 8 d after implantation by growth-associated protein-43 immunolabeling. At both time points, the number of axons regenerating into acellular grafts treated with chondroitinase was severalfold greater than in control grafts. Growth into the chondroitinase-treated grafts was pronounced after only 4 d, suggesting that the delay of axonal growth normally associated with acellular grafts was attenuated as well. These findings indicate that chondroitinase treatment significantly enhanced the growth-promoting properties of freeze-killed donor nerve grafts. Combined with the low immunogenicity of acellular grafts, the ability to improve axonal penetration into interpositional grafts by preoperative treatment with chondroitinase may be a significant advancement for clinical nerve allografting.

Key words: nerve regeneration; acellular nerve graft; chondroitinase; basal lamina; neurite inhibitor; Schwann cell

Peripheral nerve injuries are a major source of chronic disability. Although nerve tissue possesses the potential to regenerate after injury, this response is strictly dependent on regenerating axonal sprouts making appropriate contact with endoneurial basal laminae in the distal nerve segment (Fawcett and Keynes, 1990). In crush injury, where there is axonal disruption but the continuity of the endoneurial sheath remains intact, axons regenerate within their original basal lamina, and complete recovery can be expected. In contrast, axonal regrowth may be severely compromised after nerve transection, and end-to-end primary repair is variable because of misalignment of nerve sheaths, basal laminae, and axons (Dagum, 1998). These difficulties are of even greater concern in segmental repair by interpositional nerve grafting.

Nerve grafting is warranted with nerve ablation but presents several practical challenges. Over the years, various graft alternatives have been explored. Presently viewed as a developing alternative is the application of allogenic nerve grafts. Although fresh donor grafts have the difficulties of other organ replacement strategies, the importance of viable cellular elements in nerve grafts may be far less important. Although Schwann cells contribute significantly to the regenerative process, the nerve sheath structure contains the essential scaffolding and adhesive cues to promote axonal regeneration, and significant regeneration has been achieved in acellular (freeze-killed) nerve grafts (Ide et al., 1983; Hall, 1986; Gulati, 1988; Nadim et al., 1990). Moreover, acellular nerves greatly reduce the concerns of host–graft immunorejection (Evans et al., 1994, 1998). These features provide considerable promise for the use of cryostored allografts. On the other hand, the absence of viable cells precludes nerve degeneration and remodeling, which seem to promote the regenerative process (Bedi et al., 1992; Danielsen et al., 1994). Laminin is a major neurite-promoting component of the basal lamina that almost certainly represents the adhesive stimulus for successful axonal regeneration (Wang et al., 1992). Interestingly, normal nerve is rich in laminin, yet before degeneration, it is refractory to axonal growth (Langley and Anderson, 1904; Brown et al., 1994). This suggests that the growth-promoting activity of laminin is suppressed in a normal nerve environment and that laminin activity must somehow be revived in nerve degeneration and ensuing regeneration.

Increasing evidence indicates that chondroitin sulfate proteoglycans (CSPGs) can inhibit axonal growth and negate the growth-promoting activities of extracellular matrix components (Muir et al., 1989; Snow et al., 1990; Brittis et al., 1992; McKeon et al., 1995; Fidler et al., 1999). We recently found that the peripheral nerve contains CSPG, which inhibits the growth-promoting activity of endoneurial laminin (Zuo et al., 1998a). Furthermore, our work supports the conclusion that CSPG-degrading enzymes represent a mechanism by which the growth-promoting properties of laminin may be restored within a degen-

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erating nerve (Zuo et al., 1998b; Ferguson and Muir, 2000). In the present study, we examined nerve regeneration into acellular nerve grafts treated with chondroitinase. Our results indicate that degradation of CSPG improves the ability of axons to traverse the host–graft interface and significantly increases the number of axons growing into acellular nerve grafts.

MATERIALS AND METHODS

Preparation of acellular nerve grafts. Adult (180–200 gm) female Sprague Dawley rats (Harlan, Indianapolis, IN) were used as nerve donors and recipient hosts. This project was reviewed and approved by the Institutional Animal Care and Use Committee. Donor rats were anesthetized with halothane and decapitated. Sciatic nerves were exposed through a gluteal muscle-splitting incision and isolated free of underlying fascia. A 15 mm nerve segment was excised rostral to the bifurcation into common peroneal and tibial nerves. The segments were rinsed with cold sterile Ringer's solution, stabilized by pinning the ends to a thin plastic support, and transferred to a cryogenic vial. The vials were submerged in liquid nitrogen for 2 min and then transferred to a 37°C water bath for 2 min. This freeze–thaw cycle was repeated, yielding acellular nerve grafts that were then stored in liquid nitrogen. On the day before grafting, the nerve grafts were warmed to room temperature and incubated in 100 μl of PBS, pH 7.4, containing 2 U/ml chondroitinase ABC (Sigma, St. Louis, MO) or in PBS (vehicle) only for 16 hr at 37°C. The grafts were rinsed twice with Ringer's solution and kept on ice before use. The chondroitinase ABC preparation is highly purified and stated by the manufacturer to be essentially free of protease activity.

Interpositional nerve grafting. Twelve rats received bilateral acellular nerve grafts, one chondroitinase-treated and one vehicle-treated. Host rats were deeply anesthetized using xylazine (15 mg/kg, i.m.) and ketamine (110 mg/kg, i.p.). The sciatic nerve was exposed and supported by a plastic insert placed between the nerve and underlying tissue. The region of the nerve halfway between the sciatic notch and bifurcation was first coated with fibrin glue. Using serrated scissors, a 2.5 mm segment of the host nerve was excised and replaced with a freshly trimmed 10 mm acellular nerve graft. The graft was coapted to the host nerve stumps by epineurial neurorrhaphy using one 9–0 Ethilon suture at each end. Fibrin glue was then applied to stabilize the coaptations, which, in combination with the initial fibrin coating, also reduced protrusion of nerve elements (endoneurial mushrooming) (Menovsky and Bartels, 1999). The muscle was closed with 4–0 sutures, and the skin was closed with wound clips. After recovery from the anesthetic, animals were returned to standard housing. Nine rats were killed at 8 d and four at 4 d after grafting.

Animals were deeply anesthetized and decapitated. The graft and 3 mm of proximal and distal host nerve were removed and immersed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4°C. The specimens were equilibrated with PBS and immersed in 30% sucrose in phosphate buffer for 2 d at 4°C. Using a dissecting microscope and the epineurial sutures as landmarks, each specimen was subdivided into three segments representing (1) the proximal nerve–graft interface, (2) the main graft, and (3) the distal nerve–graft interface. The specimens were embedded and cryosectioned. Longitudinal sections were taken through the nerve–graft interfaces to examine the continuity of the coaptations.

The main grafts were sectioned serially on the transverse plane in a recorded measure to assess the extent of axonal growth by microscopy. Regenerating axons were labeled by growth-associated protein-43 (GAP-43) immunofluorescence (see below) in sections of the grafts at 0.56 mm intervals. Epifluorescent photomicrographs were acquired using a SPOT digital camera system (Diagnostic Instruments, Sterling Heights, MI) and Axiovert 10 microscope (Carl Zeiss, Thornwood, NY). GAP-43-positive axon profiles were scored using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

Immunocytochemistry. Axonal regeneration was assessed by GAP-43 immunofluorescence and digital image analysis. Tissue sections mounted on slides were washed with PBS and then treated with 0.5% Triton X-100 in PBS for 10 min. The sections were treated with blocking buffer (10% serum in PBS and 0.1% Triton X-100) and then incubated overnight at 4°C with primary antibodies (diluted in blocking buffer). Bound primary antibodies were labeled with swine anti-rabbit immunoglobulins (Dako, Carpinteria, CA) or goat anti-mouse immunoglobulins (Sigma) conjugated with fluorescein or rhodamine for 1 hr at room temperature in darkness. The anti-mouse secondary antibody was preadsorbed with rat serum before use. The sections were washed, post-fixed with 4% paraformaldehyde in PBS, rinsed, and coverslipped in fluorophore-stabilizing mounting media. Affinity-purified rabbit anti-GAP-43 peptide antibody was produced in our laboratory as described previously (Ferguson and Muir, 2000) and was used at 2 μg/ml. Polyclonal antibody 1918 (Chemicon International, Temecula, CA; 1:1000) binds only to the unsaturated disaccharide unit that remains attached to the linkage region of the CSPG core protein exposed by digestion with chondroitinase ABC (Bertolotto et al., 1986). Polyclonal anti-mouse laminin-1 antibody (Sigma; 1:1000) was used to label basal laminae. Polyclonal anti-S-100 antiserum (Dako; 1:500) was used to label Schwann cells. Dark-field images were inverted and optimized for printing in Photoshop (Adobe Systems, San Jose, CA).

Cryoculture bioassay. Cryoculture is a neurite outgrowth assay in which neurons are cultured directly on fresh-frozen nerve sections and was performed as described previously (Ferguson and Muir, 2000). Briefly, chondroitinase- and vehicle-treated nerve segments were sectioned at 20 μm, mounted on sterile coverslips, and stored at −20°C until used. Where indicated, sections were treated with chondroitinase ABC (0.1 U/ml) or vehicle (50 mM Tris-HCl, pH 8.0, containing 50 mM NaCl) for 2 hr at 37°C. Purified dorsal root ganglionic (DRG) neurons from day 8 chick embryos were seeded directly on the nerve sections in a defined N2 medium (Bottenstein et al., 1980) containing 10 ng/ml nerve growth factor. Cryoculture assays were terminated after 24 hr of incubation by fixation with 100% methanol. Neuritic growth by DRG neurons was accessed by GAP-43 immunofluorescent labeling. Epifluorescent photomicrographs were acquired as described for tissue sections. Neurite lengths were measured directly using Image-Pro Plus software (Media Cybernetics). At least 250 neurons with neurites greater than one cell body (~15 μm) were scored for each condition in each experiment.

RESULTS

Degradation of CSPG by treatment of acellular nerve segments with chondroitinase

We previously found that treatment of peripheral nerve tissue sections with chondroitinase ABC degrades and inactivates neurite growth-inhibiting CSPG associated with the endoneurial basal laminae (Zuo et al., 1998a). The first aim of this study was to determine whether chondroitinase treatment effectively degraded CSPG throughout intact segments of acellular nerves. Segments of rat sciatic nerve (1.5 cm in length) were made acellular by repeated freeze–thaw cycles and then bathed en bloc in a chondroitinase ABC solution for 16 hr. CSPG degradation within the chondroitinase-pretreated nerves was examined by immunolabeling with neoepitope antibody Ab1918. This antibody binds to an epitope created on the core protein after lysis of the chondroitin sulfate chains by chondroitinase ABC (Bertolotto et al., 1986). Ab1918 immunostaining was intense throughout the entire pretreated nerve segment (Fig. 1*A*). Furthermore, the intensity of Ab1918 immunostaining was not increased by an additional post-treatment of the sections with chondroitinase (Fig. 1*B*). Ab1918 immunoreactivity was absent in acellular nerves not exposed to chondroitinase (results not shown). These findings indicate that the en bloc chondroitinase treatment effectively permeated all nerve compartments and thoroughly degraded CSPG side chains.

In a normal nerve, CSPG and laminin are mainly colocalized in the nerve sheaths and basement membranes, including Schwann cell basal laminae (Zuo et al., 1998a). Their distributions were unchanged after repeated freeze–thaw, and there was no indication at the light microscopic level that en bloc chondroitinase treatment altered extracellular matrix structures (Fig. 1*A,C*). The integrity of chondroitinase-treated acellular nerve segments was an important consideration for their subsequent use as nerve regeneration grafts. Accordingly, we also examined the structural integrity of the pretreated nerve segments after nerve grafting. The intensity and distribution of Ab1918 immunoreactivity (in

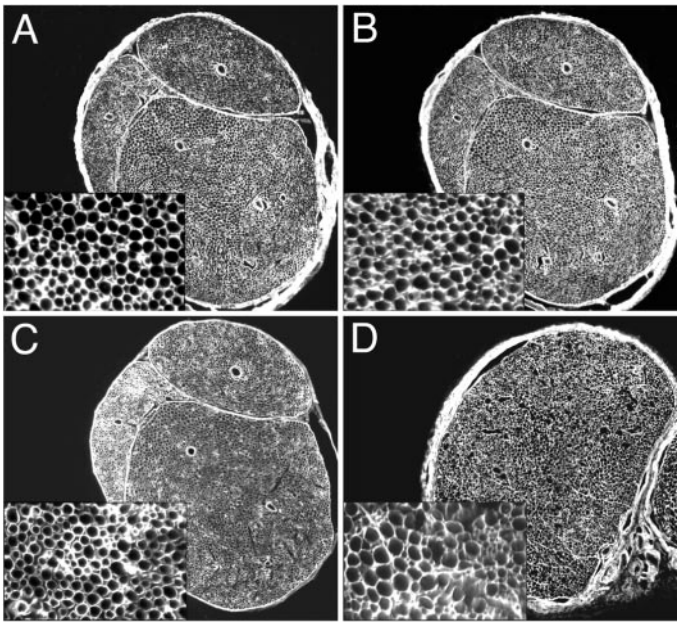

Figure 1. CSPG neoepitope immunofluorescence of chondroitinase-treated acellular nerve grafts. Acellular (freeze-thawed) rat sciatic nerve segments were treated en bloc with chondroitinase ABC for 16 hr *in vitro*. *A*, Neoepitope (chondroitinase-dependent) labeling with Ab1918 showed that en bloc treatment with chondroitinase effectively permeated all nerve compartments and degraded CSPG side chains. *B*, The intensity of Ab1918 immunolabeling was not increased by additionally treating sections of the nerve shown in *A* with chondroitinase, indicating that the initial en bloc treatment was thorough. *C*, The structural integrity of Schwann cell basal laminae in chondroitinase-treated acellular nerve segments was shown by laminin immunofluorescence. *D*, Ab1918 immunolabeling of chondroitinase-treated acellular interpositional nerve graft after 8 d *in vivo*.

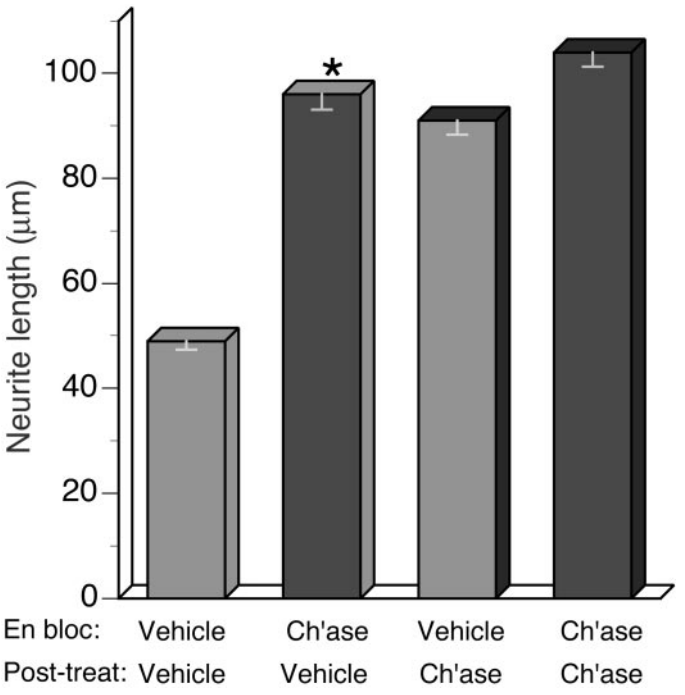

Figure 2. Inactivation of inhibitory CSPG by cryoculture bioassays of acellular nerve segments treated with chondroitinase. Acellular nerve segments were treated en bloc with chondroitinase or vehicle alone. The nerves were sectioned and then additionally post-treated with chondroitinase or vehicle only. Dissociated chick embryonic DRG neurons were grown on the nerve sections for 24 hr, and neurite lengths were scored as described in Materials and Methods. Determinations were made by scoring at least 250 neurons in each condition. Results are expressed as mean ± SEM, and statistical significance comparing the en bloc vehicle and chondroitinase conditions was determined using Student's *t* test. $^*p < 0.001$.

regions of the grafts not infiltrated by host cells) was unchanged after 8 d *in vivo*, indicating the primary structure of Schwann cell basal laminae remained intact (Fig. 1*D*). Taken together, these results demonstrate that en bloc chondroitinase treatment of acellular nerve grafts effectively degraded CSPG without compromising the basal lamina scaffold or dislocating its laminin content.

Inactivation of inhibitory CSPG by treatment of acellular nerve segments with chondroitinase

Inactivation of inhibitory CSPG in a chondroitinase-treated acellular nerve was determined by cryoculture bioassay. Embryonic chick DRG neurons were seeded onto sections of prepared nerve segments, and the neurite-promoting activity was assessed by scoring neurite growth. Results are shown in Figure 2. On sections of an acellular nerve pretreated en bloc with vehicle only, the average neurite length was 49 μm. Neurite growth on an acellular nerve pretreated en bloc with chondroitinase averaged 96 μm, representing a 95% increase compared with the control condition. To determine whether the en bloc chondroitinase treatment was thorough, cryoculture assays were performed on nerve tissues treated with chondroitinase after sectioning (post-treatment). As expected, the neurite-promoting activity of acellular nerve treated en bloc with vehicle only was increased significantly (86%) by post-treatment with chondroitinase. In contrast, chondroitinase post-treatment had only a slight additive effect on sections from en bloc chondroitinase-treated nerve grafts. These results indicate that inhibitory CSPG was effectively degraded and inactivated by bathing lengthy segments of acellular nerve grafts in small amounts of chondroitinase ABC. In addition, en bloc chondroitinase treatment effectively deinhibited the nerve grafts without disrupting the laminin-associated, neurite-promoting potential of the basal lamina scaffold. The latter point was strengthened by the observation that, as in cryoculture assays of normal and degenerated nerve (Ferguson and Muir, 2000), neurite growth on sections of chondroitinase-treated acellular nerve grafts occurred in strict association with Schwann cell basal laminae.

Nerve regeneration is enhanced by chondroitinase treatment of acellular nerve grafts

In the next set of experiments, we tested the hypothesis that chondroitinase treatment improves nerve regeneration through acellular nerve allografts. As described above, acellular sciatic nerve segments were treated en bloc with vehicle or chondroitinase ABC. Ten millimeter interpositional nerve grafts were joined to the host nerve by epineurial neurorrhaphy reinforced with fibrin glue. Each of nine host rats received bilateral grafts, one vehicle-treated and one chondroitinase-treated. Regeneration was initially examined after 8 d. First, the proximal and distal nerve–graft coaptations were examined in longitudinal sections to assess the alignment of the surgical coaptation (Fig. 3). All of the grafts were in continuity and thus were included in the

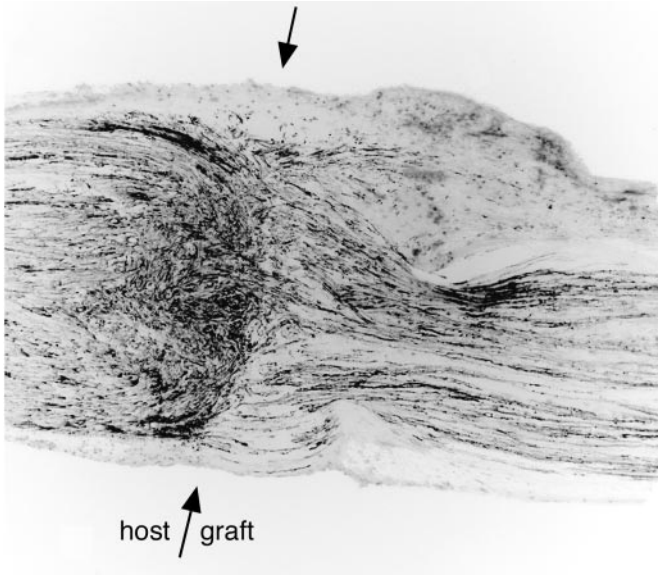

Figure 3. Assessment of the continuity and GAP-43 immunostaining of interpositional acellular nerve grafts. The continuity of each nerve graft was confirmed by examining the proximal and distal nerve–graft coaptations in a longitudinal section. At the proximal coaptation, GAP-43 labeling revealed numerous regenerating axons entering the proximal aspect of the graft. GAP-43 did not label any remnant elements within the acellular graft.

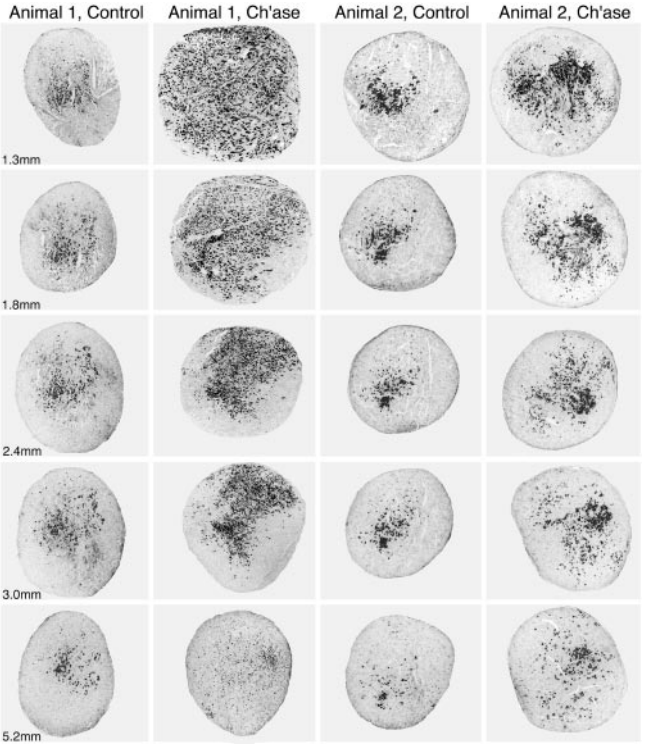

Figure 4. Axonal regeneration into acellular interpositional nerve grafts after 8 d. Shown is a representative series of sections from two animals, each receiving vehicle-treated and chondroitinase-treated grafts. Serial sections taken from the proximal graft (*top*) and subsequent 0.56 mm intervals were immunolabeled with GAP-43. In each animal receiving bilateral grafts ($n = 9$), axon growth was greater in the acellular graft treated with chondroitinase than in the vehicle-treated control. Images were cropped at the epineurium to approximate the fields scored by digital image analysis in Figure 5.

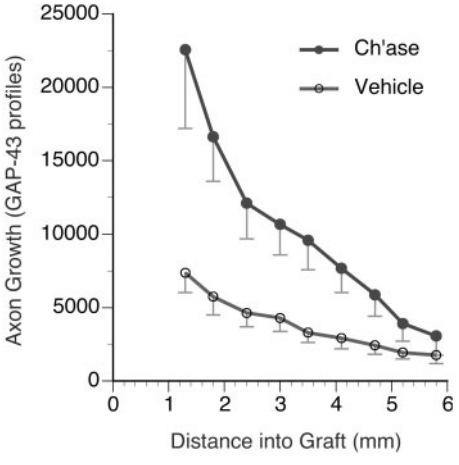

Figure 5. Greater accession of regenerating axons into chondroitinase-treated acellular nerve grafts. Serial sections of 8 d interpositional nerve grafts (as depicted in Fig. 4) were scored for GAP-43-labeled axonal profiles by digital image analysis. Data represent the mean ± SEM of nine vehicle-treated and nine chondroitinase-treated grafts assessed at the specified distances into the graft (proximal to distal).

subsequent analysis. Scoring of regeneration was based on GAP-43 immunolabling, which intensely stained growing axons. Axon and Schwann cell remnants within the freeze-killed grafts were immunonegative for GAP-43, and host Schwann cells were only very faintly stained (at an intensity below the threshold used for digital scoring). Axonal growth was assessed at specified spatial intervals within the graft by scoring GAP-43-immunopositive profiles in transverse sections. Some axonal ingrowth was observed in all grafts, as depicted in Figure 4. However, the growth into chondroitinase-treated grafts was markedly greater and more widely distributed than in control grafts. Quantitative results are shown in Figure 5. The average number of axons (GAP-43-immunopositive profiles) entering chondroitinase-treated grafts was on average more than threefold greater than in control grafts. Although the axons entering the control grafts were always restricted and most often clustered centrally, the initial growth into chondroitinase-treated grafts was more widely dispersed and especially abundant at the proximal end. These findings indicate that the success of axonal penetration into acellular nerve grafts was markedly improved by pretreatment of the grafts with chondroitinase. However, a similar number of axons was consistently observed at the distal ends of grafts in both conditions. This suggested that axonal penetration into the control grafts occurred early and then was temporally restricted, whereas axons continued to penetrate chondroitinase-treated grafts during the 8 d period.

To determine whether the latency of axonal growth into acellular grafts was reduced by chondroitinase treatment, the same analysis was performed on 4 d grafts except that the most proximal aspects of the grafts were examined and scored in a transverse section as well. Although only 3 animals receiving bilateral graphs were examined, the results were consistent with those observed for 8 d grafts. Moreover, at the most proximal aspect of the graft (0.3 mm from the host–graft interface), axonal penetration was on average fivefold greater in chondroitinase-treated grafts (Fig. 6). From these results we conclude that chondroitinase treatment decreases the latency and significantly improves the accession of axonal regeneration into acellular nerve grafts.

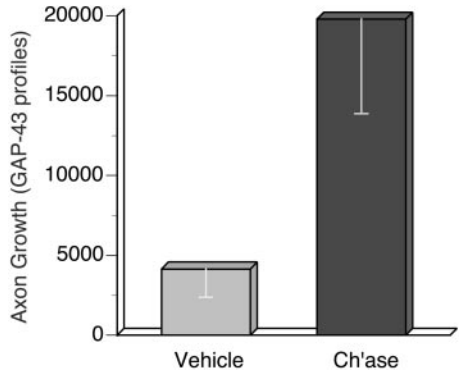

Figure 6. Axonal regeneration into the initial segment of acellular interpositional nerve grafts after 4 d. The nerve–graft interface and immediately proximal region of 4 d acellular grafts were examined as described in Figure 5. GAP-43-labeled axon profiles were compared at 0.3 mm into the grafts. Data represent the mean ± SEM of three vehicle-treated and three chondroitinase-treated grafts.

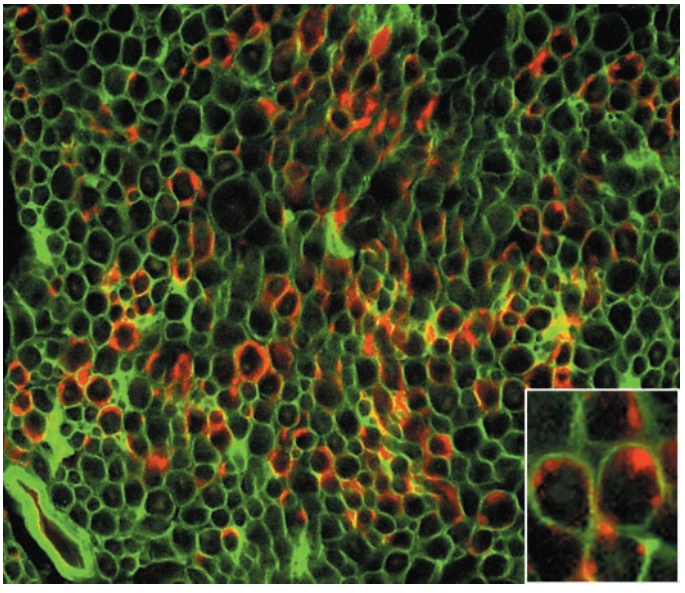

Figure 7. Axonal regeneration occurred within the basal lamina of chondroitinase-treated grafts. A transverse section from the midsection of an 8 d chondroitinase-treated acellular graft is shown double-labeled for GAP-43 (regenerating axons; *red*) and laminin (basal laminae; *green*). Multiple axons (neurites) often grew within a single endoneurial tube. Most axon profiles were found in close association with the lumenal surfaces of Schwann cell basal laminae (*inset*).

Axon regeneration occurred within basal lamina tubes of chondroitinase-treated grafts

The success of nerve regeneration depends on the growth of axons within the laminin-rich basal lamina tubes. We examined whether the association of axonal growth with basal laminae was altered by chondroitinase treatment of acellular grafts. Transverse sections of 8 d grafts were double-labeled for GAP-43 and laminin. Figure 7 represents the pattern of growth observed in the middle of a chondroitinase-treated graft. Laminin labeling was intense, and basal laminae appeared similarly intact throughout control and chondroitinase-treated grafts. Despite repeated freeze–thaw, enzyme treatment, surgical manipulation, and 8 d *in vivo*, the extracellular matrix scaffold appeared structurally intact. Multiple GAP-43-labeled axons (or neurites) were evident within individual basal laminae, and most of these were observed in close association with the lumenal surface of the tubes (Fig. 7, *inset*). A similar and minor number of neurites with ambiguous apposition were observed in control and treated grafts. By and large, the propensity of axons to grow within basal laminae was unaltered by chondroitinase treatment of acellular nerve grafts.

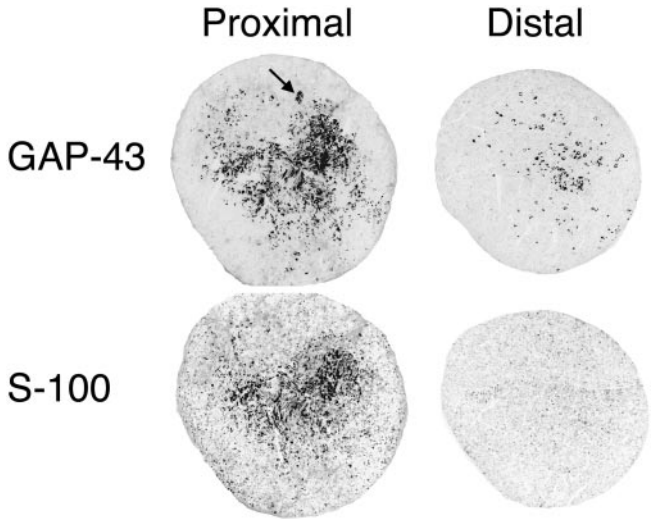

Figure 8. Association of axon regeneration and Schwann cell migration within the grafts. Serial sections of 8 d grafts were immunolabeled for GAP-43 (axons) and S-100 (Schwann cells). In proximal regions of the chondroitinase-treated grafts, Schwann cells were most often found in close association with regenerating axons. Occasional clusters of axons were observed without comigrating Schwann cells (*arrow*). At more distal points in the grafts, axons were often found without accompanying Schwann cells. Few isolated Schwann cells were intensely immunolabeled for S-100 in the more distal regions of the grafts, which contained mostly faint S-100 staining associated with freeze-killed Schwann cell remnants.

Axonal growth was not preceded by Schwann cell migration into the grafts

Serial sections of the 8 d grafts were immunolabeled for S-100 and GAP-43 to examine the migration of Schwann cells with respect to axon growth. The grafts contained two distinct patterns of S-100 staining: intense staining was associated with live, host-derived Schwann cells and faint staining with freeze-killed Schwann cell remnants. The descriptions that follow refer to the intensely stained (live) Schwann cell profiles, unless otherwise indicated. In proximal regions of the grafts, the distributions of Schwann cells and axons mainly coincided (Fig. 8). Occasional clusters of axons were found without any apparent Schwann cell association. Scattered Schwann cells were also seen in regions without growing axons. Schwann cell migration was apparent well into the 8 d grafts. However, at more distal points in the grafts, axons were most often found without accompanying Schwann cells (Fig. 8). This was confirmed in longitudinal sections including the distal coaptation (Fig. 9). S-100-labeled Schwann cells were abundant in the distal host stumps, yet few if any had invaded the distal aspect of the grafts (which contained only freeze-killed Schwann cell remnants) (Fig. 9*B*). The examples presented in Figures 8 and 9 were obtained from chondroitinase-treated grafts, and identical results were observed in the control grafts. These findings suggested that the enhancement of axonal growth in chondroitinase-treated grafts was primarily attributable to the potentiation of the neurite-promoting activity of the basal lamina.

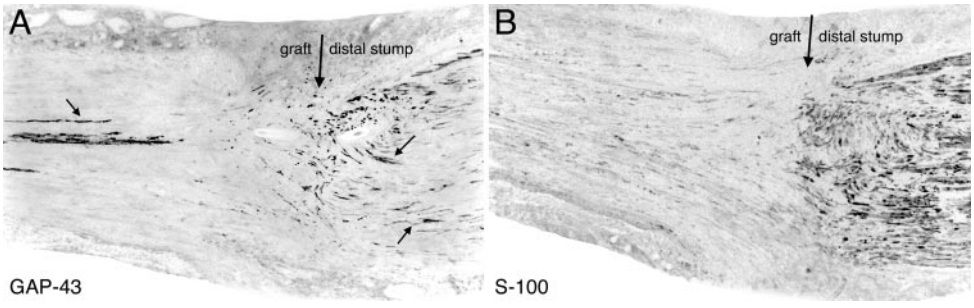

Figure 9. Axon and Schwann cell growth at the distal graft coaptation. Serial longitudinal sections of 8 d chondroitinase-treated grafts and distal nerve stumps were immunolabeled for GAP-43 (axons; *A*) and S-100 (Schwann cells; *B*). *A*, Axons (*small arrows*) approach, traverse the distal coaptation, and grow diffusely within the host distal stump. *B*, S-100-labeled Schwann cells are abundant in the distal host stumps, yet few if any invade the distal aspect of the grafts (which contains faint S-100 immunostaining associated with freeze-killed Schwann cell remnants).

In this study, the path of axonal growth was examined only in longitudinal sections of tissues immediately surrounding the proximal and distal coaptations. On entering the grafts, axon growth was directed distally, and there was no indication of deviant growth or neuroma formation within the grafts. This suggested that guidance mechanisms (or chemoattractant properties associated with the distal stump) were not compromised in chondroitinase-treated grafts. In addition, on the basis of the few instances in which axons had reached the distal extent of the graft, axons exited the grafts and continued growth into the host nerve stump (Fig. 9*A*).

DISCUSSION

The results of the present study show that (1) chondroitinase ABC treatment effectively degraded and inactivated inhibitory CSPG throughout acellular nerve segments, and (2) axonal ingress and the magnitude of axonal regeneration were markedly increased in acellular grafts treated with chondroitinase. The main conclusion is that degradation of CSPG within acellular grafts improves the ability of regenerating axons to traverse the nerve–graft interface and potentiates axonal growth within its basal lamina scaffold. These results confirm our previous *in vitro* findings that inactivation of inhibitory CSPG deinhibits the neurite-promoting activity of endoneurial laminin in normal nerves. This work supports the hypothesis that deinhibition by chondroitinase mimics an essential process that occurs naturally in nerve degeneration to reveal the growth-promoting potential of the Schwann cell basal lamina.

Our past work indicates that the conversion of a normal nerve from a nonpermissive state to one that promotes axonal regeneration may occur by degradation of the CSPG core protein by matrix metalloproteinases expressed in a degenerating nerve (Zuo et al., 1998b; Ferguson and Muir, 2000). A goal of the present study was to optimize the growth-promoting potential of acellular nerve grafts prepared from normal nerves. Although this may be achieved during nerve degeneration by metalloproteinases that degrade the CSPG core protein, inactivation of CSPG is also effectively achieved by application of chondroitinase. This bacterial enzyme is veracious, and, unlike matrix metalloproteinases, its activity appears to be unchecked by natural inhibitors in nerve tissues. An additional advantage is that this glycosidase degrades only CSPG side chains and does not disrupt nerve sheath organization or displace laminin from the Schwann cell basal lamina. Moreover, chondroitinase ABC is suggested for clinical application, and it is safe regarding adverse effects on nerve tissue and blood vessels (Olmarker et al., 1996).

Nerve grafting is warranted with nerve ablation or transection when the stumps have retracted and cannot be coapted without tension (Millesi, 1984). Presently, autologous nerve grafts are the first choice for interpositional grafting. Autografts offer distinct advantages, because they are available fresh at surgery, contain viable glial and vascular elements, and are inherently immunocompatible. However, alternatives to nerve autografts remain a goal of neurosurgeons to avoid the concurrent functional deficits associated with procuring autografts, which also preclude this approach for management of major or extensive nerve defects. Allogenic nerve grafting overcomes these concerns but remains experimental and is limited by the need for long-term systemic immunosuppression. On the other hand, recent advances have been made to reduce the immunoreactive responses to allografts by nullifying the cellular components of the graft (for review, see Evans et al., 1994). This approach may be especially relevant in light of accumulating evidence that regenerating axons can grow effectively within freeze-killed nerve grafts (Gulati, 1988; Ide et al., 1990; Sondell et al., 1998). Important characteristics of these acellular nerve grafts is that they have lost much of their immunogenicity and can be stored frozen for extended periods without losing their growth-promoting activity (Ide, 1996; Evans et al., 1998). Despite these advantages, freeze-killing also decreases the ability of grafts to promote regeneration, and acellular grafts remain inferior to those containing viable cells.

Acellular nerve grafts have been used as a model for investigating the relative contributions of the extracellular matrix and non-neuronal cells for the support of regenerating axons. It is generally agreed that both axons and Schwann cells regenerating into acellular grafts are mostly associated with preexisting basal laminae (Hall, 1986; Nadim et al., 1990; Ide et al., 1990). This association was confirmed in the present study, and we observed that axonal regeneration occurred within the Schwann cell basal laminae of chondroitinase-treated grafts as well. Axonal regrowth is accompanied by and enhanced or sustained by the ingress of Schwann cells. In our studies, Schwann cell invasion into control and chondroitinase-treated grafts appeared to follow axonal growth. Although there was a high proportion of Schwann cells in the proximal graft, Schwann cell numbers progressively diminished, and their association with axons was less evident at the distal extent of axonal growth. We also observed isolated axons and growth cones in the distal regions of the grafts, and there was little evidence of Schwann cell ingress from the distal host nerve. Otherwise, Schwann cell processes were closely associated with established axons within the grafts. More directed studies are required to determine whether chondroitinase treatment also enhanced the migration of Schwann cells into acellular grafts. Aside from the magnitude of the regenerative response, and considering the bilateral evidence that either axons or Schwann cells might have a leading role in regeneration through nerve grafts, there was no evidence that chondroitinase treatment altered the course of

axonal growth or cellular interactions commonly associated with nerve regeneration. Combined with our cryoculture studies, these findings support the conclusion that chondroitinase treatment primarily enhanced nerve regeneration in the grafts by deinhibiting the neurite-promoting properties of the basal lamina. Examination of longer nerve grafts is required to determine whether chondroitinase treatment alone is sufficient to overcome the limitations of axonal growth in acellular grafts over longer distances.

Acellular grafts prepared from fresh nerves are inherently incapable of degeneration. Schwann cells and inflammatory cells from the host nerve will migrate into and remodel the acellular graft, promoting regeneration. However, this process is slow and erratic and may be restricted in long grafts by limits in Schwann cell proliferation and migration (Hall, 1986; Anderson et al., 1991). One approach to improve the growth-promoting properties of acellular grafts is to repopulate the graft with autologous Schwann cells (Gulati et al., 1995). Autologous Schwann cell replacement strategies retain the low immunogenicity of acellular allografts and may reinstate nerve degeneration and remodeling. The importance of the degenerative process has been demonstrated in several nerve-grafting models, and fresh predegenerated nerve grafts may be as effective or more effective than fresh normal nerve grafts in promoting regeneration of peripheral nerve axons (Gordon et al., 1979; Danielsen et al., 1994). Furthermore, predegeneration reduces the initial delay of axon penetration and enhances regeneration into freeze-killed nerves as well (Danielsen et al., 1995). This indicates that, in degeneration, cellular mechanisms act to enhance the growth-promoting properties of the basal lamina, which then retains the ability to stimulate nerve regeneration after the cellular elements have been killed. Our results suggest that chondroitinase treatment mimics a key degenerative process by enhancing the growth-promoting properties of the basal lamina scaffold in acellular nerve grafts. Additionally, modification of acellular nerve grafts by chondroitinase markedly accelerates the ingress of axons and thus overcomes a major shortcoming associated with freeze-killed nerve grafts. Because the *in vivo* predegeneration of a human donor nerve is impractical, degradation and inactivation of CSPG by chondroitinase may greatly expand the clinical potential for acellular grafts.

Loss of continuity is inevitable in end-to-end nerve repair or grafting and presents a major obstacle to regenerating axonal sprouts. At the nerve-graft interface, we observed tangles of regenerating axons as they sought passage into the graft. Neuroma formation is often associated with regeneration failure after nerve injury and surgical repair (Dellon and Mackinnon, 1988). Our findings indicate that axonal penetration into acellular grafts was markedly improved by pretreating the grafts with chondroitinase. Not only did deinhibition of the Schwann cell basal laminae increase the number of axons that penetrated the grafts, but the initial delay of ingrowth appeared to be decreased as well. It is known that axon sprouts will degenerate if they fail to traverse the coaptation and make appropriate contact with basal laminae in the distal nerve or graft (for review, see Fu and Gordon, 1997). Thus, it is likely that the rapid accession into chondroitinase-treated grafts may also increase survival by axotomized neurons, which is especially important for negating the increased risk of neuronal atrophy and death associated with more proximal injuries.

Functional recovery after microsurgical nerve repair is variable. Serious sensory and motor deficits are the most likely outcomes and are the direct result of insufficient reinnervation of target organs. Any failure of initial growth into nerve grafts surely contributes to sparse reinnervation. However, even marginal increases in reinnervation can result in significant improvements in function. Combined with the low immunogenicity of acellular grafts, the ability to improve axonal penetration into interpositional grafts by preoperative treatment with chondroitinase may provide considerable improvement in clinical nerve allografting. Additionally, long-term and thorough neurological studies are required to assess the full potential of this graft preparation on recovery of function.

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